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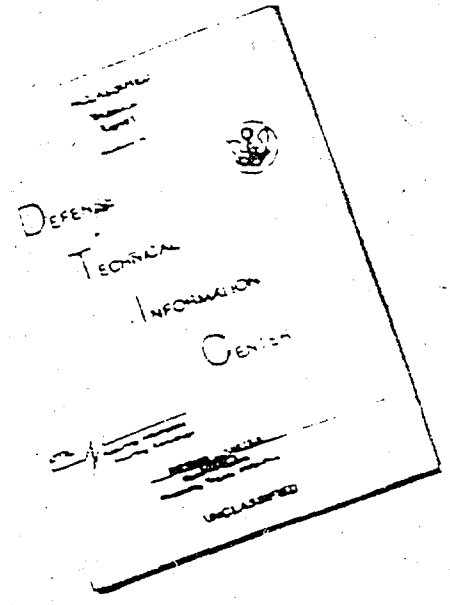

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TECHNICAL MANUSCRIPT 145

ROLE OF THE LYMPHATICS IN PATHOGENESIS OF ANTHRAX

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TECHNICAL MANUSCRIPT 145

ROLE OF THE LYMPHATICS IN PATHOGENESIS OF ANTHRAX

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ABSTRACT

Previous investigations of anthrax have indicated that the lymphatic system first becomes involved in the dynamics of the disease, and that lymph samples may be used to detect anthrax bacilli before the disease becomes apparent. The present investigations were carried out (a) to gain more information on the role of the lymphatics in pathogenesis of anthrax, (b) to determine the extent of lymphatic participation on extravascular circulation of bacilli, (c) to test for the removal of spores from the lung epithelium into the lymphatics and/or blood stream immediately after challenge or during the infectious process of the disease, and (d) to determine whether systemic anthrax might be detected earlier by observation of bacilli in the lymph than by observation of the blood.

In these studies, the thoracic and right lymph ducts of rhesus monkeys were cannulated. The organisms in the blood and lymph were compared both by dilution plate count and by direct count after the animals were infected by various routes of challenge.

Anthrax was shown to be established by the intraperitoneal, intracutaneous, or gastrointestinal routes through the lymph nodes that drain the region of challenge. In animals challenged by aerosol, the bacilli enter the lymphatics from the lung and are passed to the blood. Thereafter there is little difference in the dynamics of infection among the methods of challenge.

Although the presence of the anthrax organism can be detected in the lymph before it can be detected elsewhere in the body, the cannulation is a serious and unsure operation and should probably not be used routinely to detect the disease.

I. INTRODUCTION

Several investigators have confirmed the participation of the lymphatic system in an anthrax infection.[1-5] In order: (a) to gain more information on the role of the lymphatics in pathogenesis of anthrax, (b) to test the hypothesis on extravascular circulation of bacilli proposed by us,[6] (c) to test for the removal of spores from the lung epithelium into the lymphatics and/or blood stream immediately after challenge or during the infectious process of the disease, and (d) to determine whether detection of systemic anthrax might be made earlier by observation of bacilli in the lymph than by observation of the blood, we quantitated the change of bacilli in the thoracic lymph, right lymph, and blood following challenge with anthrax spores and during the development of a fatal septicemia. Routes of challenge were intradermal, intraperitoneal, intravenous, gastrointestinal, and via the respiratory tract.

II. METHODS

A. CANNULATION AND SAMPLING PROCEDURES

A method of cannulating the right or the thoracic lymphatic duct of the rhesus monkey* was developed and reported by Hodge and Rhian.[7] The thoracic duct, right lymphatic duct, and jugular veins were exposed by bisection of the sternum. The lymphatic ducts were cannulated and led out under the skin and out of the body on top of the head. Simultaneous cannulation of the jugular vein allowed a cannula to be placed in the heart. However, when lymphatic cannulation was not desired, the jugular vein was cannulated by a simpler operation. In less than 10 per cent of the monkeys did a common right lymphatic duct enter the external jugular vein. In the remainder the duct was either too small, located too deep in the chest to be isolated by probing, or so bifurcated and deeply embedded in fat that ligation of a common duct was not practical. A thoracic cannula was placed successfully in approximately 80 per cent of the monkeys.

Before the monkey recovered from anesthesia, it was placed in an upright position in a holding chair to prevent the cannula from being disturbed. The lymph flowed continuously and was collected in a calibrated test tube. The blood cannula and a solution of saline, glucose, or protein

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* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society of Medical Research.

was connected to a three-way stopcock. Blood was sampled when desired by inserting a syringe on the third outlet of the stopcock. After a blood sample was taken, the cannula was always back-washed immediately with a sterile solution. The system was kept sterile by inserting a cotton stopper saturated with phenol in the syringe outlet between sampling periods, and wrapping the three-way stopcock in a gauze saturated with phenol. Glucose and protein hydrolysate were given at a rate of 100 to 200 milliliters (ml) per eight-hour period, depending on the size of the monkey, to prevent dehydration and hypoproteinemia of animals in which lymph cannulae had been placed.

This method of taking samples allowed blood to be collected by one person on any schedule without contacting or exciting the animal. Although there was some restraint of the animal, lymph flow was continuous in rate of flow. Neither the surgical wound nor the host became contaminated, and the wound never became infected with anthrax from aerosol or contact sources. The volume of lymph flow was recorded at one- or two-hour intervals, and bacterial counts were done on the total volume of lymph collected during the sampling interval. Bacterial counts were made on the blood at one-, two-, three-, or four-hour intervals, depending on the experimental design and state of knowledge. Monkeys used in cannulation experiments weighed at least 12 pounds and most of them weighed 16 to 20 pounds.

B. QUANTITATION OF ANTHRAX BACILLI

The quantitative estimation of bacilli per ml of blood or lymph was done by two methods – the conventional dilution plate count, and by direct observation of a known volume distributed over a known surface. In the latter method, a standard curve to quantitate the direct observation method was developed initially by determining the correlation between the number of bacilli in a sample of blood and the number per microscopic field. Bacilli were spread (0.02 ml blood over one square centimeter (cm) surface), fixed by drying, killed by immersion for five minutes in 10 per cent formalin, and stained with Giemsa stain. By observation of the number of bacilli in some definite number of microscopic fields, the number of bacilli per ml was estimated rapidly by reference to the standard curve. A regression of direct count on plate count of $r = 0.99$ was observed in developing the standard curve. Counts of any desired accuracy were made by varying the number of microscopic fields (area) counted. In our standardized procedure we routinely count 144 fields if counts were in the range of 10^4 to 10^6 bacilli per ml and fewer fields as the count increased above that range. Using the direct count, the change in the blood or lymph was quantitatively estimated before the next two-hour sample (one hour if urgent) was taken. Dilution plates that were made at the same time as slides could not be counted until at least 12 hours after plating.

C. AEROSOL EXPOSURE TECHNIQUE

When challenge was by the aerosol route, the monkeys were exposed in an air-tight cage to an aerosol of spores of the V1b strain. The air pressure inside of the cage was made negative to the outside by attachment to a ventilated hood system. The aerosol of spores was generated in a Plexiglas cylinder (Figure 1) 28 cm long and 6.5 cm in diameter. A rubber diaphragm, which made a complete seal when placed over the monkey's mouth and nose, was mounted at one end of the cylinder. The opposite end of the cylinder was reduced to 1.9 cm to fit the inflatable end of a Darex weather balloon* that was used to collect the excess aerosol.

To begin the exposure, the cylinder, which is the aerosol mixing chamber, was placed over the monkey's nose. By a nebulizer** mounted on the side of the mixing chamber, a cloud of anthrax spores was aerosolized into the chamber. The nebulizer was operated with an air flow rate of 16 liters per minute, and the liquid spore suspension was disseminated from the nebulizer at a rate of 0.08 milliliter per minute. The concentration of spores in the nebulizer was calculated on the basis of the weight and respiration rate of the monkey. Time of exposure was four minutes and the cloud was sampled during the entire exposure period with an impinger at the rate of 7.2 liters of air per minute.

After exposure, the cylinder was removed from the monkey's nose and mouth. The collecting fluid in the impinger was removed and plated on tryptose agar. By determining from plate counts the concentration of viable spores in the collecting fluid with the volume of sampled aerosol known, it was possible to calculate the number of viable spores per liter of aerosol. The volume of air inhaled by the monkey was calculated by the Guyton formula;[8] this result was multiplied by time of exposure (four minutes) to obtain total amount of air breathed. The actual number of viable spores inhaled by the monkey was calculated by multiplying the number of viable spores in a liter of aerosol by the liters of air breathed. The procedure for aerosol exposure as well as the operation has been described on film.***

D. OTHER CHALLENGE METHODS

Intradermal challenge was over the region of the posterior tibia so that drainage to the popliteal lymph node occurred. Intraperitoneal

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* W. R. Grace and Co., Cambridge, Mass.

** Vaponefrin Standard Nebulizer, Vaponefrin Co., Portland, Oregon.

*** Film entitled "Pathogenesis of Anthrax - By Cannulation of the Lymphatics System" Library No. RH-103. Tech. Releases, Technical Information Division, Fort Detrick, Frederick, Maryland.

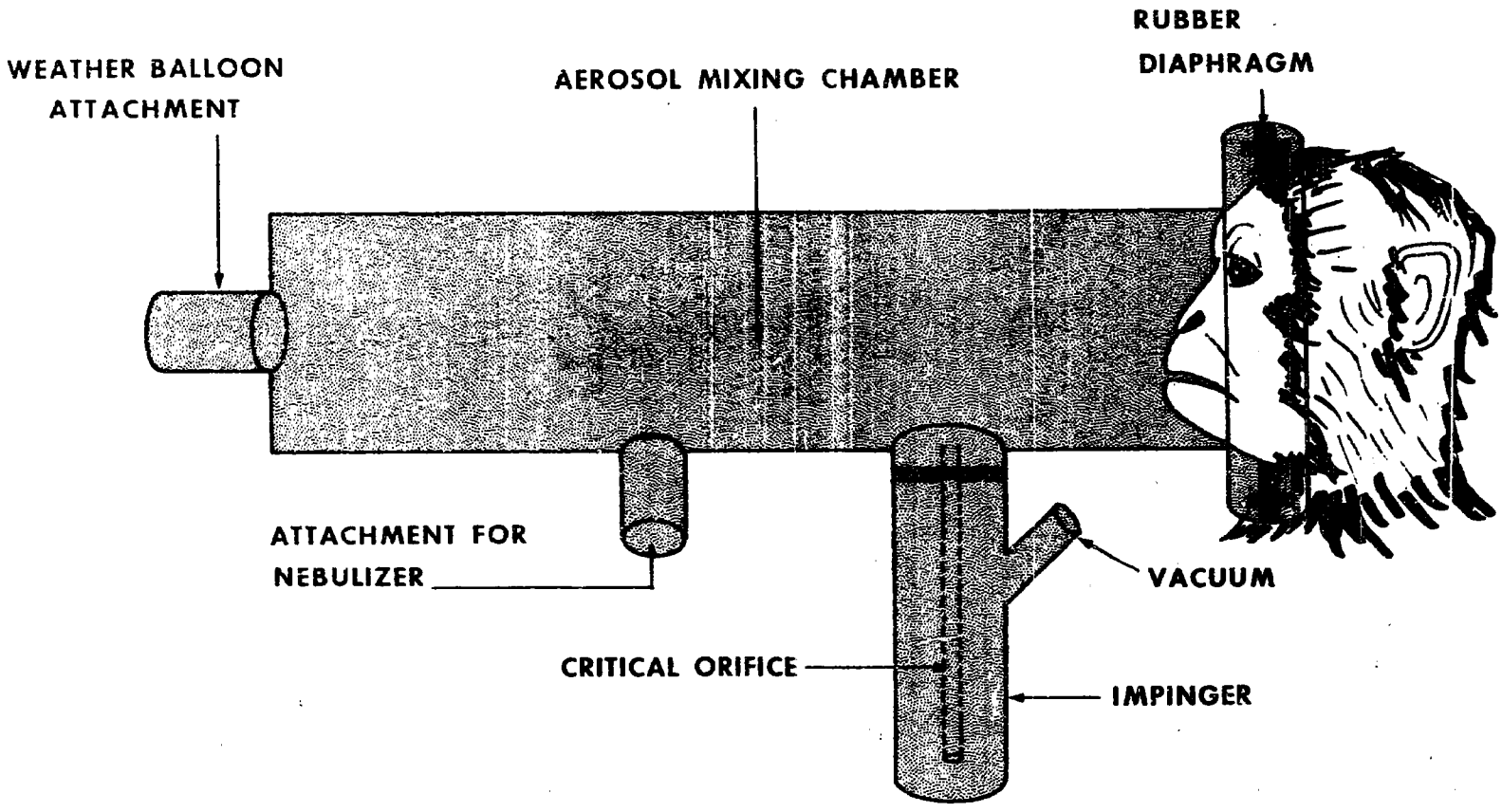


Figure 1. Aerosol Exposure Chamber.

inoculation was conventional. Intravenous challenge was through the venous cannula into the right side of the heart. The gastrointestinal challenge was made with a tube inserted into the stomach.

III. RESULTS AND DISCUSSION

A. CRITICAL OR INFLECTION POINTS IN THE PATHOGENESIS OF ANTHRAX

Although the pattern of host resistance and growth of the anthrax bacilli varies with animal species,[9] certain characteristics of *in vivo* growth occur in the dynamic spread of infection via the lymphatic system and blood stream. Certain generalizations have been made in this report to allow both presentation of the data and visualization of the dynamic nature of this disease.

Following challenge with enough spores to cause anthrax infection, a predictable series of events occur in the lymphatic system and blood stream. These critical points are initial observation of bacilli in the lymph or blood and whether bacilli are observed constantly, the number of bacilli per ml of lymph and blood, and the rate of change in numbers and death of the host. These critical points are diagrammed in Figure 2 and results following this scheme are presented in Tables I through V. Data for challenged animals are grouped according to the type of cannulation and route of challenge.

B. LYMPH FLOW RATES AND OUTPUT OF BACILLI

The anatomical variability of the right lymphatic duct experienced by Widdicombe *et al*[2] in their studies with rabbits also was found to occur in the rhesus monkey. The most simple variability was the presence or absence of a common duct. Where an anastomosis of the lymphatics occurred, the possibility existed that collateral lymphatics drained not only the lungs but some of the abdominal regions. Taking into consideration these above variables and the pathogenesis of the disease, the lymph flow as depicted in Table I can be explained on the basis of the regional lymphatic anatomy where it is considered that the right duct drains the major portion (75 per cent) of the lungs whereas the thoracic duct drains the remainder of the lungs and some of the abdominal viscera. Lymph flow was essentially constant in both the thoracic and right lymph ducts prior to anthrax septicemia (F_1, F_2, Figure 2). Flow rates remained constant until the stress of septicemia (B_2) resulted in an increase in the rate of flow of the thoracic lymph (F_2, F_3).

TABLE I. AVERAGE LYMPH FLOW AND NUMBER OF BACILLI COLLECTED IN THORACIC AND RIGHT LYMPH DUCTS DURING ANTHRAX INFECTION IN THE RHESUS MONKEY

Route of challenge	Number of animals observed	Average weight, pounds	Organisms in the blood	Lymph (average values)						Blood organisms per milliliter X 10^6
				Flow milliliter per hour		Organisms per milliliter		Organisms per hour		
				thoracic	right	thoracic	right	thoracic	right	
Intradermal	1	14	Before septicemia (F_1-F_2[a])	5.6		$3x10^7$		$2x10^8$		0[b]
			During septicemia (F_2-F_3)	6.2		$1x10^8$		$6x10^8$		0.11
Intravenous	2	14	Before septicemia (F_1-F_2)	6.8		0		0		0.000001
			During septicemia (F_2-F_3)	8.3		$1x10^7$		$1x10^8$		7.62
Intraperitoneal	1	12	Before septicemia (F_1-F_2)	10.8		$9x10^3$		$1x10^5$		0
			During septicemia (F_2-F_3)	11.2		$1x10^7$		$1x10^8$		1.6
Aerosol	2	15.5	Before septicemia (F_1-F_2)	7.6		$1x10^1$		$1x10^2$		0
			During septicemia (F_2-F_3)	13.5		$2x10^3$		$3x10^4$		0.48
	3	14	Before septicemia (F_1-F_2)		3.4		$2x10^5$		$5x10^5$	0
			During septicemia (F_2-F_3)		2.3		$7x10^6$[c]		$3x10^7$	40.0
	1	11	Before septicemia (F_1-F_2)	10.4	0.7	$2x10^0$	0	$3x10^1$	0	0
			During septicemia (F_2-F_3)	10.9	0.1	$2x10^5$	$1x10^3$	$2x10^6$	$1x10^2$	0.64

[a] See Figure 2.

[b] No organisms observed.

[c] One animal was negative.

TABLE II. ASSOCIATION OF INFLECTION POINTS AS PRESENTED IN FIGURE 2 FOR MONKEYS CHALLENGED BY THE AEROSOL ROUTE

			Initial observation (B_1, L_1) of bacteria				Inflection point in growth curve of bacteria in blood (B_2) or lymph (L_2)				Inflection point (L_3): period of static growth in lymph		Death of host and number of bacilli (B_3, L_4)		
			Hours after challenge		Organisms per milliliter		Hours after challenge		Organisms per milliliter		Hours after challenge	Organisms per milliliter	Hours after challenge	Organisms per milliliter	
Monkey number	Dose x 10^5	Type of cannulation	Blood hours	Lymph hours	Blood	Lymph	Blood hours	Lymph hours	Blood	Lymph				Blood	Lymph
1	4.0	V[a]	57		$9x10^1$		57		$7x10^1$				143	$6x10^6$	
2	5.6	V	72		$1x10^4$		74		$2x10^2$				94	$4x10^5$	
3	5.0	V	100		$2x10^4$		104		$3x10^1$				147	$4x10^4$	
4	5.0	V	56		$3x10^6$		58		$9x10^6$				64	$2x10^9$	
5	20.0	V	13		$5x10^0$		30		$6x10^2$				50	$3x10^3$	
6	6.0	V	15		$1x10^0$		33		$1x10^1$				47	$3x10^6$	
		T[b]		1		$1x10^0$		42		$1x10^0$	c				$1x10^2$
7	5.0	V	48		$9x10^4$		48		$9x10^4$				57	$2x10^6$	
		T		23		$9x10^4$		44		$2x10^1$	c				$1x10^4$
8	3.0	V	20		$3x10^2$		e						25	$4x10^1$	
		R[d]		8		$3x10^0$		e							e
9	3.0	V	26		$8x10^0$		36		$1x10^1$				58	$3x10^7$	
		R		10		$1x10^0$		32		$1x10^1$	42	$4x10^7$			$2x10^8$
10	0.5	V	34		$3x10^7$		34		$3x10^7$				47	$4x10^5$	
		R		26		$4x10^2$		26		$4x10^2$	34	$6x10^6$			$2x10^6$
11	0.1	V	26		$1x10^2$		26		$1x10^2$				44	$6x10^6$	
		T		22		$3x10^0$		26		$6x10^2$	c				$2x10^6$
		R		26		$1x10^3$		26		$1x10^3$	30	$3x10^3$			$6x10^2$
12	1.0	V	50		$4x10^2$		50		$4x10^2$				70	$9x10^5$	
		T		f											
		R		67		$4x10^5$		67		$4x10^5$	67	$4x10^5$			$3x10^5$

[a] Heart cannulation via the jugular vein.

[b] Thoracic lymph duct cannulation

[c] Death occurred before static state.

[d] Right lymph duct cannulation

[e] No buildup of organisms

[f] Lymph from thoracic duct stopped flowing 31 hrs post challenge.

TABLE III. ASSOCIATION OF INFLECTION POINTS AS PRESENTED IN FIGURE 2 FOR MONKEYS CHALLENGED BY THE INTRADERMAL ROUTE

			Initial observation (B_1, L_1) of bacteria				Inflection point in growth curve of bacteria in blood (B_2) or lymph (L_2)				Inflection point (L_3): period of static growth in lymph		Death of host and number of bacilli (B_3, L_4)		
			Hours after challenge		Organisms per milliliter		Hours after challenge		Organisms per milliliter		Hours after challenge	Organisms per milliliter	Hours after challenge	Organisms per milliliter	
Monkey number	Dose X 10^5	Type of cannulation	Blood hours	Lymph hours	Blood	Lymph	Blood hours	Lymph hours	Blood	Lymph				Blood	Lymph
1	1.0	V[a]	45		$3x10^7$		47		$9x10^6$				50	$1x10^8$	
2	1.0	V	33		$4x10^6$								38	$1x10^7$	
3	1.0	V	32		$3x10^4$		32		$3x10^4$				48	$8x10^7$	
4	1.0	V	37		$8x10^3$		37		$8x10^3$				48	$8x10^5$	
5	1.0	V	24		$2x10^4$		24		$2x10^4$				35	$3x10^7$	
6	1.0	V	30		$2x10^1$		30		$2x10^1$				42	$5x10^5$	
		T[b]		20		$7x10^3$		20		$7x10^3$	38	$2x10^8$			$7x10^7$
7	1.0	V	38		$6x10^1$		38		$6x10^1$				59	$1x10^6$	
		T		8		$2x10^1$		34		$2x10^4$	36	$8x10^4$[c]			
8	1.0	V	34		$2x10^1$		34		$2x10^1$				44	$3x10^6$	
		T		28		$6x10^5$		28		$6x10^7$	34	$2x10^7$[e]			
9	1.0	V	28		$3x10^3$		28		$3x10^3$				40	$1x10^7$	
		T		26		$6x10^0$		27		$9x10^5$	f				$3x10^7$
10	1.0	V	30		$1x10^7$		g						32	$5x10^6$	
		T		20		$1x10^6$		20		$1x10^6$	24	$1x10^7$			$1x10^4$

[a] Heart cannulation via jugular vein

[b] Thoracic lymph duct cannulation

[c] Lymph stopped flowing 21 hours before death.

[d] Not done

[e] Lymph stopped flowing 6 hours before death.

[f] Death occurred before static state.

[g] No build up of organisms.

TABLE IV. ASSOCIATION OF INFLECTION POINTS AS PRESENTED IN FIGURE 2 FOR MONKEYS CHALLENGED BY THE INTRAPERITONEAL ROUTE

			Initial observation (B_1, L_1) of bacteria				Inflection point in growth curve of bacteria in blood (B_2) or lymph (L_2)				Inflection point (L_3): period of static growth in lymph		Death of host and number of bacilli (B_3, L_4)	
			Hours after challenge		Organisms per milliliter		Hours after challenge		Organisms per milliliter		Hours after challenge	Organisms per milliliter	Hours after challenge	Organisms per milliliter
Monkey number	Dose $\times 10^5$	Type of cannulation	Blood hours	Lymph hours	Blood	Lymph	Blood hours	Lymph hours	Blood	Lymph				Blood / Lymph
1	1.0	V [a]	66		$1x10^5$		66		$1x10^5$				76	$2x10^8$
2	1.0	V	42		$1x10^5$		43		$6x10^5$				55	$8x10^7$
3	1.0	V	252		$1x10^7$		[b]						253	$9x10^6$
4	1.0	V	1		$5x10^1$		25		$3x10^1$				86	$1x10^3$
5	116.0	V	.08		$2x10^1$		13		$5x10^1$				42	$8x10^4$
6	928.0	V	2		$1x10^1$		84		$5x10^1$				128	$1x10^3$
7	10.0	V	18		$4x10^2$								20	$2x10^2$
		T [c]		11		$5x10^0$		11		$5x10^0$	16	$1x10^4$		$2x10^4$
8	11.0	V	20		$9x10^1$		20		$8x10^1$				36	$3x10^7$
		T		1		$5x10^0$		15		$2x10^1$	29	$3x10^7$		$1x10^7$

[a] Heart cannulation via the jugular vein.

[b] No build up of organisms

[c] Thoracic lymph duct cannulation.

TABLE V. ASSOCIATION OF INFLECTION POINTS AS PRESENTED IN FIGURE 2 FOR MONKEYS CHALLENGED BY THE INTRAVENOUS ROUTE

			Initial observation (B_1, L_1) of bacteria				Inflection point in growth curve of bacteria in blood (B_2) or lymph (L_2)				Inflection point (L_3): period of static growth in lymph		Death of host and number of bacilli (B_3, L_4)		
			Hours after challenge		Organisms per milliliter		Hours after challenge		Organisms per milliliter		Hours after challenge	Organisms per milliliter	Hours after challenge	Organisms per milliliter	
Monkey number	Dose X 10^5	Type of cannulation	Blood hours	Lymph hours	Blood	Lymph	Blood hours	Lymph hours	Blood	Lymph				Blood	Lymph
1	1.0	V[a]	56		$2x10^4$		64		$2x10^3$				98	$6x10^6$	
2	10.0	V	1		$3x10^0$		60		$2x10^2$				142	ND[b]	
3	100.0	V	.5		$6x10^1$		2		$7x10^0$				48	$4x10^5$	
4	114.0	V	1		$1x10^1$		22		$1x10^1$				35	$3x10^8$	
5	1000.0	V	.5		$5x10^2$		21		$4x10^1$				67	ND	
6	1140.0	V	1		$1x10^2$		19		$3x10^0$				60	$7x10^5$	
7	130000.0	V	1		$3x10^7$		28		$7x10^3$				46	$2x10^7$	
8	570000.0	V	1		$2x10^5$								38	ND	
9	0.1	V	20		$1x10^4$		19		$1x10^4$				27	$1x10^7$	
		T[c]		d		d		d		d					d
10	0.1	V	.5		$4x10^2$								23	$2x10^1$	
		T		d		d		d		d					d
11	0.1	V	32		$3x10^3$								32	$3x10^3$	
		T		d		d		d		d					d
12	3.0	V	10		$1x10^0$		18		$4x10^0$				31	$4x10^7$	
		T		22		$1x10^5$					30	$7x10^1$			d
13	1000.0	V	10		$7x10^1$		10		$7x10^1$				20	$4x10^7$	
		T		14		$5x10^2$					e				$4x10^5$
14	1000.0	V	30		$6x10^5$		31		$1x10^6$				40	$2x10^6$	
		T		14		$1x10^6$		31		$9x10^6$	36	$3x10^7$			$1x10^9$
15	1000.0	V	26		$1x10^5$		26		$1x10^5$				32	$2x10^7$	
		T		24		$5x10^5$		26		$5x10^5$	e				$5x10^7$

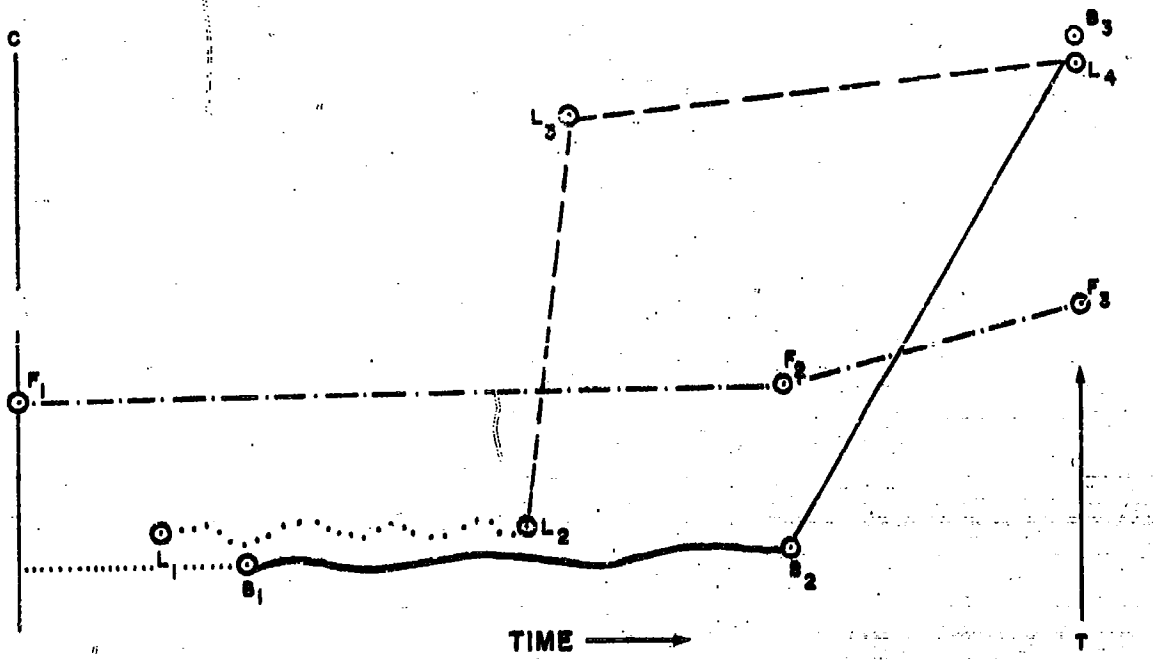
[a] Heart cannulation via jugular vein.

[b] Not done

[c] Thoracic lymph duct cannulation.

[d] No organisms observed.

e. Death occurred before static state.



"LEGEND"

C = TIME OF CHALLENGE

T = TIME OF DEATH

F = RATE OF LYMPH FLOW

F_1 - F_2 RATE CONSTANT

F_2 - F_3 RATE INCREASING

L = ORGANISMS IN LYMPH

L_1 = INITIAL DETECTION

L_2 = INFLECTION POINT INITIATING RAPID RISE TO NEAR MAXIMUM NUMBER, L_2 - L_3

L_3 = INFLECTION POINT INITIATING PERIOD OF NEAR STATIC GROWTH, L_3 - L_4

L_4 = NUMBER OF ORGANISMS AT DEATH

B = ORGANISMS IN BLOOD

B_1 = INITIAL DETECTION

B_2 = INITIATION OF SEPTICEMIA

B_3 = NUMBER OF ORGANISMS IN TERMINAL BLOOD

B_2 - B_3 = PERIOD OF LOGARITHMIC INCREASE OF BACILLI IN BLOOD (SEPTICEMIA)

Figure 2. Schema Illustrating Critical or Inflection Points in the Dynamic Course of Anthrax in a Host.

All animals having the right lymph duct cannulated were challenged by the aerosol route. Flow rates of the right lymph duct decreased approximately 27 per cent while the rate of thoracic lymph flow increased 44 per cent during the septicemic phase of the disease (F_2, F_3). All other challenge routes showed an increase of thoracic lymph during anthrax septicemia (Table I). The intravenous route increased about 18 per cent while the intraperitoneal and intradermal increased only 7 per cent.

The rate of flow before (F_1, F_2) and during anthrax septicemia (F_2, F_3) was also shown to affect lymphatic output of anthrax bacilli. The average number of bacilli per ml in the lymph and the total number of bacilli collected in the lymph before and during anthrax septicemia are given in Table I. The latter value was calculated from the mean number of organisms per ml times the average lymph flow per hour. The bacilli in the lymph were increased approximately two logs or higher during the septicemic phase of the disease (F_2, F_3). This increased output of bacilli from the lymph was partly associated with lymph output, because thoracic lymph had a 44 per cent increase of lymph during septicemic anthrax.

In lymph from the right duct, bacilli were present in a two-log increase in concentration and thereby compensated for the 27 per cent decrease in lymph flow from this duct during the same phase of the disease. We also found, however, that the lymphatic concentration of organisms was on the average equal to or greater than the concentration of bacilli in the blood during any given phase of the disease. Too few cannulated animals were quantitated to show possible significant differences between the challenge routes and lymphatic output of organisms from each duct. Until after the initial rise of organisms in the lymphatics, the total number of bacilli appeared variable by all the challenge routes, which possibly reflects, in part, the individual resistance of each animal to the disease. However, after the initial increase in number of anthrax organisms in the lymph, the total number of bacilli appeared constant by all routes of challenge. Thus, it seems the lymphatic system supports the growth of anthrax bacilli at a rate independent of route of challenge.

C. ROLE OF THE LYMPHATICS IN ANTHRAX INFECTION FOLLOWING AEROSOL CHALLENGE

The schema given in Figure 2 shows that the role of the lymphatic system in the pathogenesis of respiratory anthrax does not conflict with Rose[4] who observed that bacilli enter from the lung via the lymphatics. Bacilli were not observed in the blood (B_1) prior to their observation in the lymph (L_1) as might be expected if bacilli penetrated the capillaries directly from the lung alveoli. Although tests were made for heat-resistant spores, none were found in either the blood or lymph at any time; however, this test admittedly does not tell the state of the bacterial cell when it enters the body.

The lymphatic system appears to allow a significant exchange of bacilli between the lymph nodes and the blood. If this conclusion were not true, then as noted by Widdicombe et al,[2] cannulation of both the thoracic and right lymphatic ducts would prevent or at least delay the establishment of anthrax if the lymphatics were the only route of entry from alveoli into the body. We were able to obtain one animal (Monkey 11, Table II) in which both the right and thoracic ducts were cannulated and in which lymph flowed freely throughout the course of disease. This animal was challenged by the respiratory route. Anthrax was established and time to death (B_3) was not noticeably changed by the cannulation. Thus, it appears that significant lympho-venous connections exist.

Additional evidence for believing there are lympho-venous connections, possibly pathological in the sense of Malek et al,[6] occurred following stoppage of lymph flow (Table III, Monkeys 7 and 8). In these cases, the number of bacilli per ml of the blood (B_2 - B_3) immediately increased approximately one log, reflecting the increased number of bacilli entering the blood of pathological lympho-venous channels in the lymph nodes.

We interpret our data to show: (a) that the lymph nodes are highly efficient collectors of the bacilli since intermittent bacilli were rarely observed through a long incubation period (L_1 - L_2), but a rapid buildup occurred (L_2 - L_3) after the initial observation (L_1) of bacilli in the efferent lymph, and (b) that the lymph nodes are overcome successively one by one, since the overflow of bacilli into the blood at both the thoracic and right lymph duct increased to nearly a maximum (L_3) within a few hours following initial observation. The increase in numbers was too rapid to be explained by simple growth as with a septicemia (B_2 - B_3) and is best explained as a none-or-all flow occurring after the last lymph node was overcome.

The growth dynamics were those of a continuous culture system and would occur if a more or less constant amount of liquid entered the lymphatic system per unit of time and the same amount were removed. The dynamics would be affected if rate of flow, generation time of the bacilli, or resistance of the host changed markedly.

Our data show that the rate of lymph flow was not affected during the course of disease until septicemia. Growth of the bacilli was in equilibrium with the milieu and flow and since the latter two conditions are essentially constant, the number of bacilli per ml of effluent also was constant. These data make it appear probable that the architecture of the lymph node and lymphatic vessels is not changed appreciably by infection. This is in agreement with Berdjis et al[10] who noted little or no disturbance of architectural pattern of the lymph nodes even though they might contain anthrax bacilli. Although Trnka et al[1] did not give rates of flow in their report on sheep, they too observed a rapid buildup of bacilli in the efferent lymph to a level that continued throughout the course of disease. The two observations appear to be parallel.

Other workers experimenting with pathogenic agents have noted that whether or not disease is established is determined in the first few hours after challenge. A similar conclusion can be drawn for anthrax. The first lymph node of the series of nodes on the lymphatics draining the lungs is challenged during any unit of time by a few bacilli without a detectable capsule, i.e., dormant spores, germinated spores, or newly developed vegetative bacilli. The primary challenge continues (a) until the lung is cleared, a process that with anthrax requires weeks, or (b) until death from anthrax. With further growth of the bacteria in the lymph node, lymph vessel, or phagocyte, encapsulated bacilli develop that are relatively resistant to destruction by the phagocytes. Once the lymph nodes are overcome, tremendous numbers of bacilli are poured into the blood. Observations taken during an eight-hour period before septicemia (B_3) revealed that approximately 80 million bacilli were collected in the lymph of the cannulated monkeys (B_2). The remarkable efficiency of the reticulo-endothelial system as a filter of bacteria was demonstrated by the fact that during this period only an incipient bacteremia was observed ($B_1 - B_2$) in either uncannulated or lymph-cannulated monkeys.

After overflow of bacilli through the thoracic and right lymph ducts into the venous blood (L_2), the bacilli are distributed throughout the body, and, as noted above, retained by the reticulo-endothelial system. With few exceptions, once the septicemia (B_2) was observed the number of bacilli increased logarithmically ($B_2 - B_3$) with one doubling (apparent generation time) per 48 minutes. The concentration of bacteria in the blood of uncannulated (i.e., control) monkeys at death (B_3) averaged $10^{8.8}$ ($10^{8.5}$ to $10^{9.2}$) bacilli per ml. The variability in the terminal concentration reflects variation in the resistance of the host to anthrax toxins,[9] and also it would be expected that there would be a summation of toxin concentration multiplied by time of acting on the host.

Elsewhere we[11] have shown that anthrax toxins cause death of the rhesus monkey and that toxins can be shown under certain conditions to increase in concentration in the lymph for many hours before death. Toxins can be demonstrated in the terminal blood of most individual and all species of animals dying of anthrax thus far tested. We find a negative correlation between time of death and either the concentration of toxins in the terminal blood or number of bacilli per ml at death. King and Stein[12] have shown that vegetative cells are virtually nontoxic; therefore, toxins appear to be the principal constituents elaborated by the anthrax bacilli that cause death. Not only do anthrax toxins cause death, but we[13] suggest that a time-concentration effect of these toxins on the phagocytic system may be a primary factor in determining the time of initiaton of the terminal septicemia (B_2).

D. ROLE OF LYMPHATICS IN ANTHRAX INFECTION FOLLOWING INTRADERMAL OR INTRAPERITONEAL CHALLENGE

When anthrax is established after intraperitoneal or intracutaneous challenge, it appears from our data as well as from the data of others that the regional lymph nodes draining the site receiving the spores become infected. Depending on dose, bacilli may or may not appear in the thoracic lymph (L_1) immediately after challenge. Then a period of incubation occurs in which no bacilli are collected in the thoracic lymph, and shortly before the terminal septicemia (B_2) can be directly observed, the thoracic lymph becomes and remains positive (L_2 - L_4) for bacteria, which enter the blood stream through the thoracic duct. Basically, once the bacteria move into the lymphatics, there is little or no difference in the dynamics of infection by any of the routes of challenge—aerosol, intradermal, intraperitoneal, or gastrointestinal (Tables II, III and IV).

E. EXTRAVASCULAR CIRCULATION OF BACILLI AND ROLE OF LYMPHATICS FOLLOWING INTRAVENOUS CHALLENGE

The dynamics of the infection in animals challenged by the intravenous route are similar to those of animals challenged by other means (Table V). However, this route furnishes information on possible extravascular circulation of bacilli through the lymphatics that was not furnished by the other routes of challenge. Following challenge by all routes except intravenous, bacteria were detected in the thoracic lymph (L_1) some hours before a septicemia (B_2) was observed. Since the intravenous route of challenge failed to show this phenomenon when challenge was at relatively low doses (Monkeys 9, 10, 11, Table V) that consistently established anthrax, the critical experiment to verify that extravascular circulation of anthrax bacilli occurs was negative. Thus, our hypothesis presented in an earlier paper,[13] that extravascular circulation of bacilli through the lymphatic would be a feature of infection, was not supported by these data. It seems very probable that anthrax cells with a volume of at least four cubic microns may be too large to follow this route of circulation. Tests with other particulate materials of smaller size, as viruses, may show that the hypothesized extravascular circulation does occur.

In addition to the above reported data, monkeys were also challenged by the gastrointestinal route. This type of challenge was performed to determine whether spores from the aerosol challenge route that reached the enteric region caused foci of infection that resulted in the establishment of anthrax. Spores deposited on the ciliated surfaces of the lung alveoli constitute 65 to 75 per cent of the retained spores from an aerosol challenge, and most of these spores are passed into the stomach within a few hours.[14] Two monkeys each were challenged with spore doses of 10^2, 10^4, 10^6, and 10^8, respectively by means of a K-31 sterile infant feeding tube*

* Pharmaseal Laboratories, Glendale, California.

inserted directly into the stomach. After challenge the tube was rinsed with distilled water and withdrawn. All monkeys challenged enterically survived regardless of dose, indicating that infection by the enteric route from spores deposited on the ciliated membranes of the lung and in the head and mouth does not appear to be likely but does remain a remote possibility.

E. EARLY DETECTION OF SYSTEMIC ANTHRAX

Experimental cannulation of the lymphatics is a feasible method of detecting generalized anthrax earlier than by observation of a septicemia. However, considering: (a) the seriousness of the operation, (b) the possibility that cannula cannot be placed, and (c) the biological variability of hosts, cannulation is not considered a practical method of early detection.

F. GENERAL COMMENTS

Because the lymph was collected and not returned to the donor, the dynamics of infection as studied in this report will tend to extend the time-to-death.

The prolonged use of protein hydrolyzate and glucose in preventing hypoproteinemia is thought to change the osmolarity of the blood. This was remedied by substituting human plasma at the same rate lymph was being removed from the body. Fortified dextran* with potassium and magnesium was also effective but inferior to the human plasma. Bovine serum was tried with poor results. Monkeys, after the first injection of bovine serum, became violently ill with ensuing vomiting, respiratory distress, and finally death.

IV. SUMMARY

In order to determine whether systemic anthrax might be detected earlier by observation of the lymph than of the blood, and to better understand the role of the lymphatics in the pathogenesis of anthrax, the thoracic and right lymph ducts of rhesus monkeys were cannulated. The organisms in the blood and lymph were compared after different routes of challenge.

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* Abbott Laboratories, North Chicago, Illinois.

After challenge via the aerosol route, initial entry of bacilli from the lung occurs primarily through the lymphatics to the blood. A bacteremia was never observed prior to the observation of bacilli in the lymph. Following a challenge of about 500,000 spores, bacilli usually were observed in the thoracic lymph at about 20 hours. A bacteremia was observed later usually closely followed by a fulminating septicemia. Anthrax was established while the right and thoracic lymph was drained from the animal, indicating there is a significant exchange of bacilli directly from the lymphatics to the blood.

Lymph nodes were highly efficient collectors of anthrax bacilli as lymph tended to be either sterile or, after the initial observation of bacilli in the efferent lymph, to build up very rapidly in number of bacilli per ml of lymph. These observations indicate that lymph nodes are overcome successively. Once the rapid buildup of organisms in the lymph occurs, the growth dynamics are similar to those of a continuous culture system at equilibrium state. The rate of thoracic lymph flow was constant until septicemia then tended to increase in rate of flow, suggesting that the architecture of the lymph node and vessel is not changed appreciably by infection. An average of 80 million bacilli were generated in the infected lymphatics and poured into the blood over an 8 hour period before a septicemia occurred.

When anthrax is established by intraperitoneal, intracutaneous or gastrointestinal challenge, the regional lymph nodes draining the region of challenge become infected. Thereafter, there is little or no difference in the dynamics of infection from that described following challenge by the aerosol route. After challenge by all routes except intravenous, bacilli were detected in the thoracic lymph, approximately 12 hours before detection in the blood. Because bacilli failed to be found in the thoracic lymph before building up in the blood after intravenous challenge with a low dose, extravascular circulation (i.e., from blood to lymph) of anthrax bacilli appears not to occur. In spite of the fact that cannulation of the lymphatics allows detection of systemic anthrax before it can be identified in the blood, the seriousness of the operation to cannulate the thoracic lymph and the inherent morphological and physiological variation of individual hosts precludes routine use of this technique in diagnosis of anthrax. Anthrax was not established following gastrointestinal challenge of eight monkeys.

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